

PHYLOGENY AND BIOGEOGRAPHY OF *CHAMAECYPARIS*
(CUPRESSACEAE) INFERRED FROM DNA SEQUENCES
OF THE NUCLEAR RIBOSOMAL ITS REGION

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ABSTRACT. Phylogenetic analyses of the five extant species of *Chamaecyparis* (Cupressaceae) based on nrDNA ITS sequences yield a single most parsimonious tree in which *C. pisifera* (Japan) and *C. formosensis* (Taiwan) form a clade that is sister to a clade including *C. lawsoniana* (western North America) and *C. obtusa* (Japan and Taiwan) plus *C. thyoides* (eastern North America). Dispersal-vicariance analyses (DIVA) suggest a minimum of two dispersal events between eastern Asia and North America.

Key Words: phylogeny, biogeography, *Chamaecyparis*, eastern Asian–North American disjunct, nrDNA ITS

The floristic similarities between eastern Asia and North America (EA-NA) have attracted the attention of systematists and biogeographers since the nineteenth century (Axelrod et al. 1998; Boufford 1998; Boufford and Spongberg 1983; Graham 1999; Gray 1846, 1859; Hong 1993; Li 1952; Manos and Donoghue 2001; Tiffney 1985a, b; Wen 1999; Wu 1983). Our current understanding is that there was a more homogeneous mesophytic “boreotropical flora” in the early to middle Tertiary around the Northern Hemisphere. The modern biogeographic pattern resulted from differential loss of species in different geographic regions in response to orogenic and climatic changes in the late Tertiary (Axelrod 1960; Axelrod et al. 1998; Chaney 1947; Guo 1999; Manchester 1999; Qian and Ricklefs 1999; Stewart and Lister 2001; Tiffney 1985a, b; Wolfe 1975). However, this explanation does not provide much insight into the pathways of migration and patterns of diversification of plant lineages around the Northern Hemisphere.

Recently, molecular analyses have shed light on phylogenetic patterns in plant clades that are disjunctly distributed between eastern Asia and North America (Wen 1999). Xiang et al. (1998) summarized results from phylogenetic studies of seven plant genera with such a disjunction, and concluded that there was a congruent pattern wherein species of western and eastern North America form a clade relative to Asian species. However, when a molecular clock was used to estimate divergence times in these and other lineages, a wide range of divergence times was inferred, indicating that multiple vicariant events, at different times, might have resulted in a similar phylogenetic pattern (Xiang et al. 2000). This is known as “pseudocongruence” (Cunningham and Collins 1994). With the analysis of more clades, additional phylogenetic patterns have come to light, and the range in disjunction times has likewise expanded (e.g., Donoghue et al. 2001; also see Li et al. 2001a, b). Nonetheless, the number of clades that have been investigated is still very limited.

Chamaecyparis Spach is a disjunct genus between eastern Asia and North America (Li 1952). Plants of *Chamaecyparis* are small to large evergreen trees with branchlets in flat sprays bearing scale-like leaves in four rows. Their cone scales are peltate, subglobose, and bear two seeds. Recent phylogenetic analyses indicate that *Chamaecyparis* belongs to a well-supported clade, the Cupressoideae of the Cupressaceae (Brunsfeld et al. 1994; Gadek et al. 2000). *Chamaecyparis* contains five species (Farjon 1998), two of which are endemic to the United States: *C. thyoides* (L.) Britton, Stearns & Poggenb. in the east from Maine to Florida and westward to Mississippi, and *C. lawsoniana* (A. Murray) Parl. in the western States including southwestern Oregon and northern California (Vidakivic 1991). There are three species in eastern Asia: *C. formosensis* Matsum. endemic to Taiwan, *C. pisifera* (Siebold & Zucc.) Endl. endemic to Honshu and Kyushu of Japan, and *C. obtusa* (Siebold & Zucc.) Endl. endemic to central and northern Taiwan, and to Honshu, Kyushu, and Shikoku of Japan (Hwang et al. 2001; Iwatsuki et al. 1995). The Taiwan plants of *C. obtusa* have sometimes been segregated as a separate species, *C. taiwanensis* Masam. & Suzuki; we treat these here as *C. obtusa* var. *formosana* (Hayata) Hayata.

The objectives of the present study were (1) to reconstruct phylogenetic relationships of *Chamaecyparis*, and (2) to elucidate its historical biogeography based on the inferred phylogeny. We chose to use sequences of the nrDNA ITS region because such data have been used successfully in resolving phylogenetic relationships of other conifer lineages (Gernandt and Liston 1999; Li et al. 2001a, b; Liston et al. 1999).

Table 1. Specimens used in this study. AA = Arnold Arboretum.

Taxon & JLI DNA number	Voucher & Locale	GenBank #
<i>Chamaecyparis formosensis</i> 2751	Hwang, S.-Y. s.n., Taiwan	AY211257
<i>C. formosensis</i> 2752	Hwang, S.-Y. s.n., Taiwan	AY211258
<i>C. lawsoniana</i> 2702	AA 1164-71B, Ore., U.S.A.	AY211253
<i>C. lawsoniana</i> 2753	AA 1625-83B, Ore., U.S.A.	AY211254
<i>C. obtusa</i> 2705	AA 13038A, Japan	AY211251
<i>C. obtusa</i> 2706	AA 447-38A, Japan	AY211250
<i>C. obtusa</i> var. <i>formosana</i> 2701	AA 833-69B, Taiwan	AY211252
<i>C. pisifera</i> 2707	AA 22799A, Japan	AY211256
<i>C. pisifera</i> 2708	AA 1067-38A, Japan	AY211255
<i>C. thyoides</i> 2709	AA 13047A, Ala., U.S.A.	AY211248
<i>C. thyoides</i> 2710	AA 13047C, Ala., U.S.A.	AY211249
<i>Fokienia hodginsii</i> 2730	AA 1562-80A, Fujian, China	AY211260
<i>F. hodginsii</i> 2749	Li, J., Guangdong, China	AY211259

MATERIALS AND METHODS

Thirteen samples were included in this study, representing all species of *Chamaecyparis*; *Fokienia hodginsii* (Dunn) A. Henry & H. H. Thomas was included for rooting purposes because of its well-supported sister relationship with *Chamaecyparis* (Brunsfeld et al. 1994; Gadek et al. 2000). For each species we sampled two accessions; in the case of *C. obtusa* we included two accessions from Japan and one from Taiwan to represent var. *formosana* (Table 1).

We extracted DNAs from silica-gel dried or fresh leaf material using a Qiagen DNeasy Plant Mini Kit (cat. # 69104, Germantown, MD). Polymerase Chain Reactions (PCR) were conducted to amplify the nrDNA ITS region in a Perkin-Elmer thermocycler using primers ITS4 (White et al. 1990) and ITS-LEU (Baum et al. 1998). The Qiagen PCR purification kit (Santa Clarita, CA) was used to clean PCR products for direct cycle sequencing using an ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit (Foster City, CA). For each sample the ITS region was sequenced using three primers including ITS4, ITS-LEU, and 5.8Sgym (Liston et al. 1996). Sequences were read using an ABI Capillary Genetic Analyzer 3100 (Applied Biosystems, La Jolla, CA) and were then edited using Sequencher (3.0, GeneCode Inc., Ann Arbor, MI). Sequence alignment was done manually. Sequence boundaries of the ITS-1, 5.8S, and ITS-2 regions were determined by comparing published sequences of *Calocedrus* Kurz in the GenBank (AF287248).

Parsimony analyses were carried out with PAUP* (Swofford 2002, version 4.0 b10) using branch and bound tree search with default

options. Characters were equally weighted and their states were unordered. Gaps were treated as missing data. Bootstrap analyses using 500 replicates were conducted to evaluate support for individual clades (Felsenstein 1985).

Three biogeographic processes—dispersal, vicariance, and extinction—are responsible for the formation of modern geographic distribution (Morrone and Crisci 1995). In dispersal-vicariance analysis, costs are assigned to particular events and areas are inferred for ancestral nodes in a phylogenetic tree so as to minimize the sum of these costs (Ronquist 1997). This method has been used successfully in elucidating biogeographic patterns of families and genera of angiosperms in the Northern Hemisphere (Manos and Donoghue 2001). To infer ancestral areas and migration pathways in *Chamaecyparis* we implemented the dispersal-vicariance analysis using the computer program DIVA 1.1 (Ronquist 1996), using the default event costs (0 for vicariance, 1 for both dispersal and extinction). Three areas of endemism were recognized in these analyses: eastern North America (ENA), western North America (WNA), and eastern Asia (EA), the latter including Mainland China, Taiwan, and Japan.

RESULTS

The alignment generated a data set of 1,134 characters, 198 of which were parsimony informative. The length of the 5.8S was 146 base pairs in all species except for *Chamaecyparis obtusa* var. *formosana*, whose 5.8S was one base pair shorter. The ITS-1 region was 3.5 times as long as the ITS-2. Sequence divergences between *Fokienia hodginsii* and species of *Chamaecyparis* ranged from 6–12.6%, while those within *Chamaecyparis* were from 4.3–12.8%.

Parsimony analyses produced a single tree of 134 steps (Figure 1; CI = 0.88, RI = 0.9). The accessions of each species clustered together. In the case of *Chamaecyparis obtusa*, the Taiwan accession was united with one of the Japanese specimens to the exclusion of the other. The basal split within *Chamaecyparis* separated a *C. pisifera*–*C. formosensis* clade (bootstrap = 91%) from a clade containing *C. lawsoniana*, *C. thyoides*, and *C. obtusa* (bootstrap = 77%). *Chamaecyparis thyoides* and *C. obtusa* appear to be more closely related to one another (bootstrap = 73%) than either is to *C. lawsoniana*.

Dispersal and vicariance analyses identified two equally parsimonious ancestral area assignments for *Chamaecyparis* (Figures 2a, b): (a) the composite of all three areas (EA, WNA, ENA), and (b) eastern Asia

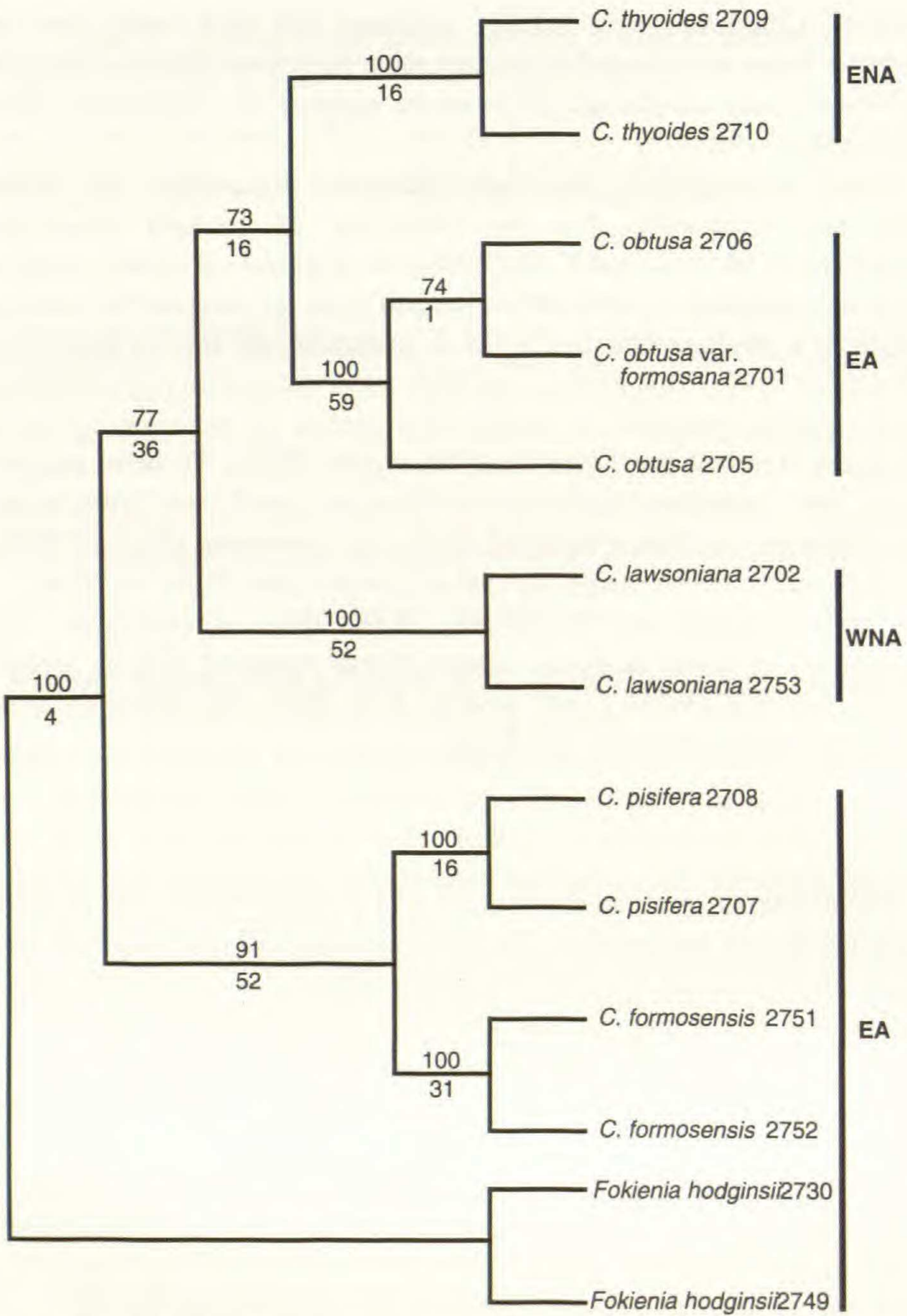


Figure 1. The single most parsimonious tree generated for *Chamaecyparis* using sequences of nuclear ribosomal DNA ITS region (134 steps; CI = 0.88, RI = 0.9). *Fokienia hodginsii* was included for rooting purposes. Numbers above and below the branches are bootstrap percentages and number of base substitutions, respectively. Acronyms denote geographic distribution. ENA = eastern North America, EA = eastern Asia, WNA = western North America.

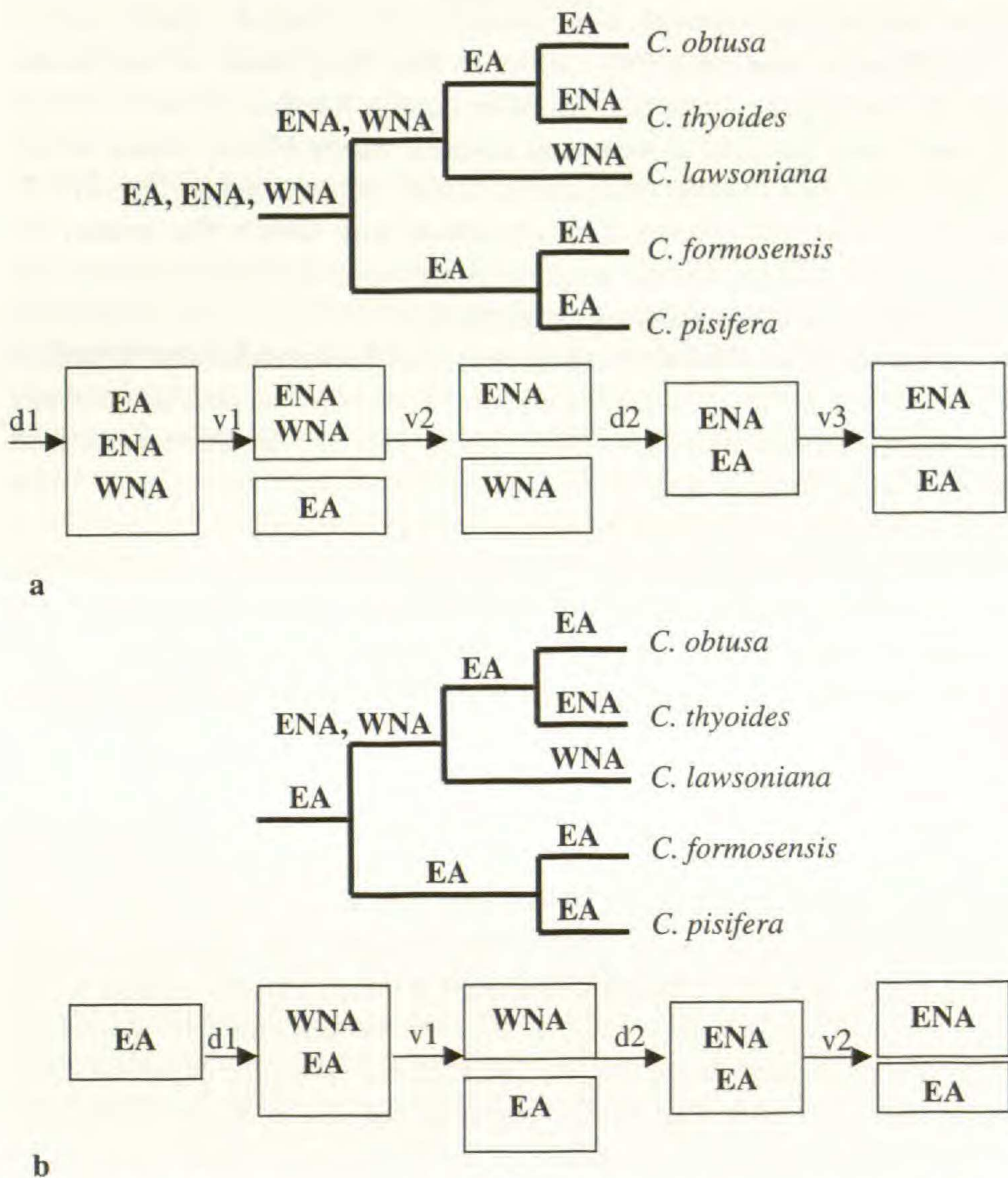


Figure 2. Alternative scenarios from DIVA analyses, showing inferred ancestral areas and vicariance and dispersal events (see text for discussion). Dispersal events are denoted by d1 and d2, and vicariance events by v1, v2, and v3. Areas of endemism are denoted by EA (eastern Asia), ENA (eastern North America), and WNA (western North America).

(EA). In both cases two dispersal events were inferred to explain the modern distribution pattern. When the maximum number of areas assigned to an ancestral node was constrained to two, EA was inferred to be the ancestral area. Again, two dispersal events were required (Figure 2b).

DISCUSSION

In *Chamaecyparis* the ITS-1 is more than three times as long as the ITS-2. This appears to be true for other conifer lineages (Li et al. 2001a, b), but is not the case in most angiosperms where ITS-1, whose length ranges from 187–298 bp, is equal to, or only slightly longer than ITS-2, which is from 187–252 bp long (Baldwin et al. 1995). The reason for this remains unclear, but the longer ITS region in conifers provides more informative sites for resolving relationships.

As noted above, *Chamaecyparis obtusa* in Taiwan has been treated as *C. obtusa* var. *formosana* (Cheng and Fu 1978), or sometimes as a separate species, *C. taiwanensis* (Liu 1966). In our ITS tree the three accessions of *C. obtusa* form a clade within which the Taiwan accession is more closely related to one of the Japanese accessions than it is to the other.

Glands are conspicuous on the upper side of the leaves in all species of *Chamaecyparis* except for *C. obtusa*. Our results imply that leaf glands are a plesiomorphy, and were probably lost in *C. obtusa*.

In a number of disjunctly distributed plant genera, the Asian species and the North American species form separate clades (e.g., Li et al. 2001b; Liston et al. 1999; Xiang et al. 2000). This has sometimes been assumed in making statistical comparisons of species richness and diversification between eastern Asia and North America (Guo and Ricklefs 2000; Qian and Ricklefs 2000). However, this pattern is not universal (Donoghue et al. 2001). For example, the Japanese species *Hamamelis japonica* (Hamamelidaceae) is more closely related to the North American species (*H. virginiana*, *H. vernalis*, and *H. mexicana*) than it is to another Asian species, the Chinese *H. mollis* (Li et al. 2000; Wen and Shi 1999). The North American species of *Taxus* (Taxaceae) form a clade with the exception of *T. canadensis*, which appears to be more closely related to the Eurasian species (Li et al. 2001a). In *Chamaecyparis*, it appears that the eastern North American species, *C. thyoides*, is more closely related to the eastern Asian *C. obtusa* than it is to the western North American *C. lawsoniana*. Therefore, no matter where the group originated, more than one biogeographic event was involved in the formation of the modern distribution pattern. Our unconstrained DIVA analyses suggest two alternatives, both entailing two dispersal events (Figure 2). In the first (Figure 2a), the ancestor of *Chamaecyparis* became widespread in eastern Asia (EA), western, and eastern North America (WNA, ENA) via dispersal (d1). This was followed by a vicariance event (v1) that created an EA line containing modern *C. formosensis* and *C. pisifera*, and an NA line. Subsequently,

within North America the western and eastern populations were separated (v2). Finally, the ENA species spread to Asia (d2), followed by vicariance (v3). In the second alternative, which is preferred when the number of areas occupied by ancestral species is limited to two, the first species of *Chamaecyparis* lived in EA. This species spread to WNA (d1) and a subsequent vicariance event (v1) resulted in a WNA and an EA species. This Asian species later spread to ENA (d2), and another vicariance (v2) then resulted in modern *C. thyoides* and *C. obtusa*.

At present we see no strong reason to prefer one scenario over the other. However, additional data could potentially sort this out. First, additional sampling within species could reveal a pattern that favors one scenario over the other. For example, analyses including additional samples within *Chamaecyparis thyoides* might indicate that some of its populations are more closely related to *C. obtusa*, suggesting dispersal from ENA to EA, as in Figure 2a. Alternatively, finding some *C. obtusa* populations more closely related to *C. thyoides* would suggest dispersal from EA to ENA, favoring the Figure 2b scenario. Second, evidence on the distributions and ages of fossils could help. For example, the scenario depicted in Figure 2a implies that *Chamaecyparis* was present in ENA early in the evolution of the group, whereas ENA was occupied only later in the Figure 2b scenario. There have been reports of fossil leaves and seed cones of both *Chamaecyparis* and *Fokienia* from the upper Cretaceous and early Paleocene (Bell 1957; McIver and Basinger 1989, 1990; Tao 1992). However, it is difficult to reliably relate these fossils to extant species of *Chamaecyparis*, and hence they have not been included in our DIVA analyses. Edwards (1984, 1992) examined morphological differences among the modern species of *Chamaecyparis* and used interspecific differences to assign fossils to the extant species. According to his analysis both *C. thyoides* and *C. lawsoniana* were present in western North America by the early to middle Eocene (43–58 MYA; Edwards 1984). This seems to support the first scenario. However, this explanation may be erroneous for the following two reasons. First, *Chamaecyparis* has a much more ancient history than the Eocene (Bell 1957; McIver and Basinger 1989, 1990; Tao 1992) and there exists the possibility that North American species are derivatives from EA. Second, so far we have not found fossils that are widespread and represent the common ancestor of species of *Chamaecyparis*. Nevertheless, Edwards' (1984) findings at least suggest the extinction of *C. thyoides* in western North America. Clearly, much more attention is needed for precise phylogenetic placement of fossils of *Chamaecyparis* from both NA and EA.

Our DIVA analyses, albeit inconclusive, indicate that the geographic history of *Chamaecyparis* has been complex, involving several intercontinental migration events at different times. In general, this is consistent with previous findings of movement in both directions and at several times (e.g., Donoghue et al. 2001; Xiang and Soltis 2001; Xiang et al. 2000; Schultheis and Donoghue, unpubl. data). Additional molecular data (such as chloroplast DNA sequences) would test the phylogenetic hypothesis, and, together with the placement of the fossil material, would provide the possibility of inferring divergence times.

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